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Intratumoral injection of an adenovirus expressing interleukin 2 induces regression and immunity in a murine breast cancer model

(cancer therapy/expression vectors)

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ABSTRACT **Rodent tumor cells engineered to secrete cytokines such as interleukin 2 (IL-2) or IL-4 are rejected by syngeneic recipients due to an enhanced antitumor host immune response. An adenovirus vector (AdCAIL-2) containing the human IL-2 gene has been constructed and shown to direct secretion of high levels of human IL-2 in infected tumor cells. AdCAIL-2 induces regression of tumors in a transgenic mouse model of mammary adenocarcinoma following intratumoral injection. Elimination of existing tumors in this way results in immunity against a second challenge with tumor cells. These findings suggest that adenovirus vectors expressing cytokines may form the basis for highly effective immunotherapies of human cancers.**

Many tumor cells express various peptide antigens on their cell surface in association with major histocompatibility class I molecules (MHC I), and these antigens may allow immune effector cells to distinguish tumor from normal tissue (reviewed in ref. 1). Many of these tumor antigens have been isolated and shown to be recognized by human cytotoxic T lymphocyte (CTL) cell lines (2, 3) or tumor-infiltrating lymphocytes (TILs) (4, 5). In addition, synthetic peptide epitopes displayed on the surface of tumor cells in association with MHC I molecules result in specific antitumor CTL activity (6). Nevertheless, tumor cells known to express potentially antigenic peptides manage to evade host immunosurveillence and proliferate *in vivo* (2, 3, 7). TILs are usually present in tumor biopsies but are unresponsive to the tumor antigens and fail to mediate cytotoxic activity (8). It has been suggested that in some instances tumor cells may lack secondary costimulatory signals required to induce expression by immune effector cells of cytokines necessary for activation and proliferation of CTLs (9, 10). This can result in anergy and thus failure of the T cells to respond to the antigen in a subsequent exposure. Indeed, it has been shown that CTL-mediated rejection of the tumor could be induced by transfection of melanoma tumor cells with DNA encoding the B7 costimulatory molecule (11). In the absence of costimulatory signals, it may be possible to bypass this requirement by providing exogenous cytokines, the downstream effect of costimulation, and overcome or prevent anergy of the immune effector cells.

Many cytokines can mediate antitumor activity *in vitro* and *in vivo* (summarized in ref. 12). Interleukin 2 (IL-2) has been demonstrated to possess antitumor activity through its ability to stimulate the cell-mediated killing activity of CTLs (13), to induce lymphokine-activated killer cells (14, 15), and to activate TILs (16, 17). Systemic delivery of recombinant IL-2 has been used in animal models and in the clinic and has met with some success (reviewed in ref. 18). However, the short half-life of IL-2 in serum requires repeated high doses, resulting in severe side effects including vascular leak syndrome, edema, anemia, fevers and chills, nausea, and hypotension (reviewed in ref. 19). To avoid these problems, local delivery of low doses of IL-2 has been investigated in animal models and was found to mediate antitumor activity, resulting in increased survival and reduced tumor growth without the side effects associated with high dosing regimens (20, 21). As a more effective method of achieving high intratumoral concentrations of IL-2, attention has turned to the use of gene delivery systems to express IL-2 continuously within or around the tumor. Transduction of tumor cells with retrovirus vectors expressing the IL-2 gene has been shown to reduce the tumorigenicity and metastatic potential of B16 melanomas (22), CMS-5 fibrosarcoma (23, 24), and the MBT-2 bladder carcinoma (25). However, certain problems associated with the use of retroviral vectors (e.g., low titers, stability) have led us to examine adenovirus (Ad) vectors for immunotherapy of cancer.

The utility of adenoviruses (Ads) as gene therapy vectors is currently being intensively investigated (26–30). The ability to produce large quantities of purified virus with relative ease makes this system very attractive for clinical use. Moreover, for immunotherapy of cancer, Ad vectors have many advantages over other commonly used systems. Deletion of the early region 1 (E1) genes renders the virus replication deficient and combining E1 deletions with deletion of the nonessential E3 region results in Ad vectors with a capacity of up to 8 kb of foreign DNA (31). The vectors can infect a wide variety of replicating and nonreplicating cell types and efficiently express genes linked to appropriate promoters. More important, Ad DNA does not normally integrate into the infected cell genome, and thus expression of the foreign gene product is typically transient. When used to deliver cytokines in immunotherapy, an Ad-based vector would therefore not result in chronic stimulation of the immune system as might occur following administration of vectors capable of integration. We have constructed an E1/E3-deleted recombinant Ad vector that expresses human IL-2 (hIL-2) under control of the human cytomegalovirus immediate early promoter (HCMV IE) and the simian virus 40 poly(A) signals (SV40 An). This vector was found to express high levels of hIL-2, modulate tumorigenicity, and induce regression of existing tumors leading to protective immunity in a transgenic mouse model of mammary adenocarcinoma.

MATERIALS AND METHODS

Construction of Recombinant Plasmids and Viruses. Plasmids were constructed according to standard protocols (32). The cDNA for hIL-2 was inserted between *Xho* I and *Eco*RI

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Abbreviations: IL, interleukin; Ad, adenovirus; h, human; CMV, cytomegalovirus; SV40, simian virus 40; pfu, plaque-forming unit(s); PyMidT, polyoma middle T antigen; CTL, cytotoxic T lymphocyte; TIL, tumor-infiltrating lymphocyte.

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sites in the polylinker region of the shuttle plasmid pCA14 (33) to create pCAIL-2P. After purification by alkaline lysis (34) and cesium chloride density gradient banding, pCAIL-2P was cotransfected into 293 cells (35) with pBHG10 DNA (31) to generate AdCAIL-2 (see Fig. 1).

Cells and Viruses. Cell lines used include the following: MRC5, human fibroblast strain (ATCC CCL 171); 793, vertical phase human melanoma (36); MeWo, metastatic human melanoma (36); Wm35, radial phase human melanoma (37); MCF7, human breast carcinoma (ATCC HTB 22); FVBMT, primary murine polyoma middle T antigen (PyMidT)-induced mammary adenocarcinoma explanted from transgenic mice (this paper); B16BL6, murine melanoma (38); 293, adenoviral E1 transformed human embryonic kidney cells (35); and 293N3S, 293-derived spinner cells (39). All cell culture media and supplements was obtained from GIBCO. All viruses were grown and titered in 293 cells except for cesium chloride banded stocks of virus, which were grown in 293N3S spinner cells.

Detection of IL-2 Expression. Cells were infected at a multiplicity of infection of 10 plaque-forming units (pfu) per cell and at various times postinfection aliquots of infected cell supernatant were removed, quick frozen in a dry-ice/ethanol bath, and stored at −70°C for detection of IL-2. Levels of secreted hIL-2 were quantitated using the Quantikine ELISA kit (R & D Systems).

Preparation of Tumor Cells from Transgenic Animals. The transgenic mice used as a model for mammary adenocarcinoma in this study [strain MT#634 (40)] possess the PyMidT expressed under the control of the mouse mammary tumor virus long terminal repeat. These mice develop spontaneous adenocarcinomas of all mammary epithelium by 8–10 weeks of age.

Tumor-bearing transgenic mice were sacrificed and the tumors were explanted, minced, and incubated at 37°C with gentle stirring in collagenase/dispase solution [25 mg of collagenase and 250 mg of dispase (both from Boehringer Mannheim) in 100 ml of phosphate-buffered saline (PBS)]. Large clumps of cells were allowed to settle and the supernatant was centrifuged at 1500 rpm for 5 min at 4°C in a Beckman GPR centrifuge to pellet the cells. The pellets were resuspended in minimal essential medium (supplemented with penicillin/streptomycin and L-glutamine) and 10% fetal bovine serum, and cells were plated at 10^7 per 150-mm tissue culture dish. After an overnight incubation to allow the cells to adhere, the tumor cell cultures were rinsed twice with PBS, fresh medium was added, and the cells were incubated for a further 24 hr.

***In Vitro* Infection of Tumor Cells.** Cells were rinsed twice with PBS and infected with cesium chloride banded viral stocks (41) of either AdCAIL-2 or Addl70-3 [an E1-deleted control virus (31)] at 100 pfu per cell or were mock infected. Following viral adsorption at 37°C for 30 min, the growth medium was replaced and the cells were incubated 20–24 hr and then harvested by trypsinization, centrifuged, and resuspended in PBS at 10^6 cells per 200 μl. Syngeneic FVB/N mice (Taconic Farms) were anesthetized with isofluorane (Abbot) and injected s.c. in the right hind flank with 200 μl of tumor cell suspension. Mice were then monitored visually and by palpation for time of tumor onset.

Intratumoral Injections of Ad Virus. Tumor cell cultures were prepared as described above but were not infected with virus. After incubation for 48 hr, the cells were harvested and 10^6 cells were injected s.c. into syngeneic mice. After 21 days, when palpable tumors had developed in all animals, the mice were injected intratumorally with 5×10^8 pfu of AdCAIL-2 or Addl70-3 in 100 μl of PBS or with PBS alone. Tumors were measured using calipers prior to injection of virus and at weekly intervals thereafter. Tumor size was estimated by determining the longest diameter and average width and calculating the volume assuming a prolate spheroid.

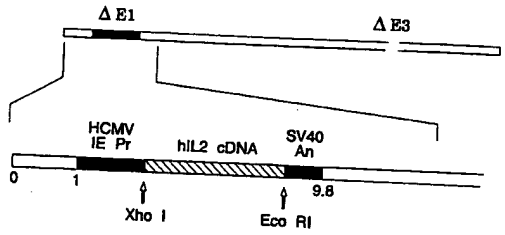

FIG. 1. Structure of AdCAIL-2. Plasmid pCAIL-2P (not shown) was constructed by ligating the hIL-2 cDNA flanked by *Xho* I and *Eco*RI sites (from pABS.A2, Chiron Corporation) into the polylinker region of pCA14 (33) placing the cDNA hIL-2 under the control of the HCMV IE promoter and the SV40 An signal. Cotransfection of 293 cells with pCAIL-2P and pBHG10 generated the recombinant virus AdCAIL-2. The HCMV IE Pr and the SV40 An (black boxes) are shown flanking the hIL-2 cDNA (hatched box). Ad5 sequences with corresponding mu indicated below are represented by the open boxes.

RESULTS AND DISCUSSION

Kinetics of IL-2 Expression in Cells Infected with AdCAIL-2. The structure of AdCAIL-2 is illustrated in Fig. 1. The vector has deletions of E1 and E3 sequences and a substitution of E1 by an expression cassette containing the HCMV IE promoter, the hIL-2 cDNA, and the SV40 An signals. Cells infected with AdCAIL-2 produced two polypeptides of 15 kDa and 17 kDa, recognized by hIL-2-specific monoclonal antibodies in Western blots of infected cell extracts and culture supernatants. IL-2 produced by infected cells was found to be biologically active as demonstrated by proliferation of the IL-2-dependent cell line CTLL-2 in bioassays (data not shown). IL-2 production following AdCAIL-2 infection of a variety of different cells was quantified by ELISA (Fig. 2). Most human cell lines were found to produce and secrete 1–2 μg of IL-2 per 10^6 infected cells over a period of 3–4 days, whereas expression by murine cells was 50- to 100-fold lower. The differences in expression between murine and human cell lines are likely due at least in part to the relatively lower activity of the HCMV IE promoter in murine cells compared to human cells (C.L.A. and F.L.G., unpublished). The levels of IL-2 produced, in the ng range, should nonetheless be sufficient for biological activity in treated animals.

***In Vivo* Modulation of Tumorigenicity.** To determine whether expression of IL-2 by cells transduced with AdCAIL-2 could promote antitumor activity *in vivo* we chose to study a transgenic mouse model of mammary adenocarcinoma. Breast cancer affects one in nine women in North America and is the leading cause of death in nonsmoking women (42). Although primary tumors can usually be surgically removed, the out-

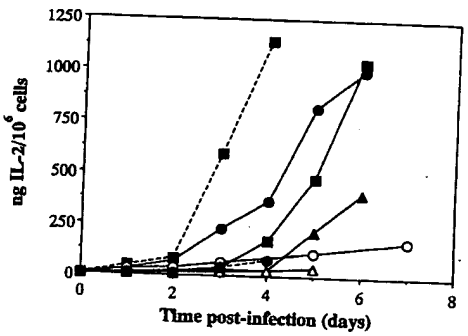

FIG. 2. Expression kinetics of AdCAIL-2. Levels of IL-2 secreted by AdCAIL-2-infected cells (multiplicity of infection of 10 pfu per cell) were determined by ELISA. Cell lines: MRC5 (■, dashed line), 793 (●), MeWo (▲), Wm35 (■), MCF7 (●, dashed line), FVBMT (○), B16BL6 (△).

come of current therapies is often unsuccessful in preventing metastases (43) and 50% of women developing primary malignancy will eventually die from metastatic disease (44). A majority of breast cancers have been shown to overexpress various oncogenes, such as c-*neu* (erbB-2) (reviewed in ref. 45), and these molecules may be potential tumor antigens by which immune effector cells can distinguish tumor tissue from normal tissue. Breast cancer is therefore a prime target for treatment by immunotherapy whereby immune responses triggered against the tumor may result in the establishment of memory immune effector cells that recognize and destroy tumor cells and may prevent metastatic disease.

The transgenic mice possessing the PyMidT under the control of the mouse mammary tumor virus long terminal repeat develop adenocarcinoma of all mammary epithelium by 8–10 weeks of age. These tumors resemble the scirrhous carcinomas found in human breast cancers (45), are heterogeneous, and will metastasize to the lungs. Cells from primary tumors can be explanted from the transgenic mice and treated with a collagenase/dispase solution to generate single cell suspensions that can be maintained in culture. Because the tumor cells express PyMidT they should be relatively immunogenic and, consistent with this, delivery of two consecutive doses of irradiated tumor cells i.p. could induce protection against lung metastases when live tumor cells were subsequently delivered by tail vein injection (T.B. and J.G., unpublished). Although PyMidT should be a potential target for recognition by specific CTLs the cells are not rejected following injection of 10^6 tumor cells s.c. into unimmunized syngeneic animals, and palpable solid tumors develop in 100% of injected animals by 15–20 days postinjection. The tumors continue to grow and have never been observed to regress spontaneously. This system therefore provides a good model of mammary carcinogenesis in humans and an excellent model in which to test the effect of cytokine expression on the immune response to immunogenic tumors that evade antitumor responses.

In initial studies on the effect of AdCAIL-2 on tumorigenicity of PyMidT tumor cells, the cells were explanted and infected *in vitro* with either AdCAIL-2 or Addl70-3 [an E1-deleted control virus containing no insert (31)] at 100 pfu per cell or were mock infected. After an additional 24 hr, by which time AdCAIL-2-infected cells had begun to express IL-2 (Fig. 2), syngeneic animals were injected s.c. with 10^6 infected tumor cells and monitored for tumor development (Fig. 3). There was no significant difference in tumor onset in animals injected with mock-infected versus Addl70-3-infected cells, but a delay of 3–5 weeks was seen for animals receiving AdCAIL-2-infected cells. However, 100% of animals in all groups eventually developed tumors. Viral infection of tumor cells (with either Addl70-3 or AdCAIL-2) did not inhibit cell growth or proliferation *in vitro*, as determined by [^{3}H]thymidine incorporation assays (data not shown), suggesting that the delay in tumor onset was not a result of cell killing due to viral infection or IL-2 production but was most likely due to IL-2-induced immune responses that were able to delay, but not prevent, tumor growth.

Regression of Established Tumors upon Intratumoral Injection. The ability of AdCAIL-2 infection to modify tumorigenicity led us to investigate the effect of local production of IL-2 in a more clinically relevant setting. Syngeneic animals were given 10^6 PyMidT cells s.c. and tumors were allowed to grow for 21 days, at which time all animals had developed palpable tumors ≈ 10 mm^3 in size. Tumors were then directly injected with 5×10^8 pfu of AdCAIL-2, Addl70-3, or with PBS and mice were monitored for tumor growth (Fig. 4*A*) and survival (Fig. 4*B*). Tumor volume, measured as described in *Materials and Methods*, is shown as a function of time after virus injection in Fig. 4*A*. Intratumoral injection with Addl70-3 resulted in a slight inhibition of tumor growth compared to

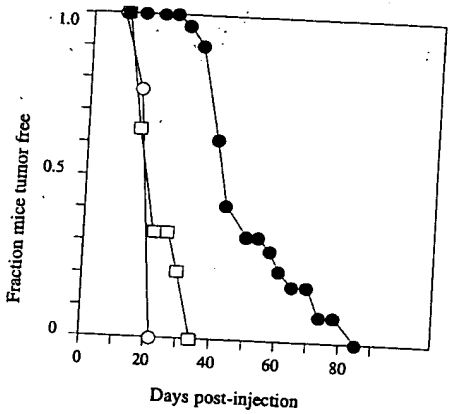

FIG. 3. Transduction of PyMidT tumor cells with AdCAIL-2 results in a delay in tumor onset. Tumor-bearing transgenic mice were sacrificed and the tumors were explanted and grown in tissue culture dishes overnight. Cells were then infected with either AdCAIL-2 or Addl70-3 at 100 pfu per cell or were mock infected. Syngeneic FVB/N mice were injected s.c. in the right hind flank with 10^6 tumor cells. Mice were monitored for the onset of tumor formation and the percentage of mice remaining tumor free is shown as a function of time postinjection. The graph represents the pooled data from two independent experiments. ○, Uninfected, $n = 16$; □, Addl70-3 infected, $n = 9$; ●, AdCAIL-2 infected, $n = 19$.

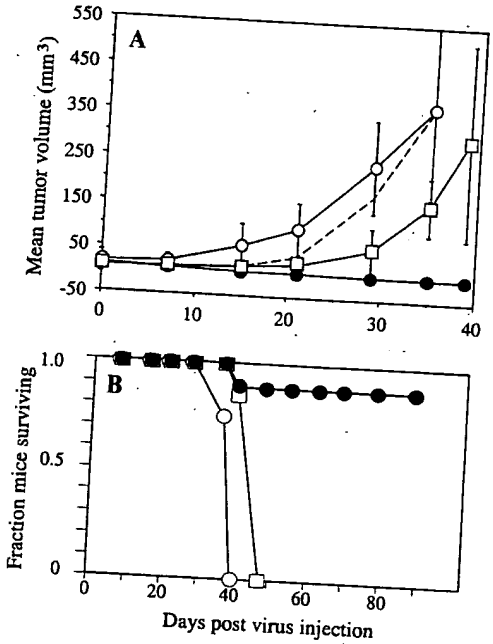

FIG. 4. Tumor regression following intratumoral injection of AdCAIL-2. Tumor cells from transgenic mice were explanted and cultured for 24 hr, at which time 10^6 tumor cells were injected s.c. into syngeneic mice. Twenty-one days postinjection of tumor cells, when palpable tumors had developed in all animals, the mice were injected intratumorally with 5×10^8 pfu of AdCAIL-2 or Addl70-3 or with PBS alone. (*A*) Tumors were measured prior to injection of the virus and then weekly (see text). ○, PBS, $n = 4$; □, Addl70-3, $n = 6$; ●, AdCAIL-2, $n = 8$. Tumor volume for the single AdCAIL-2-injected animal that failed to respond is indicated by the dashed line. (*B*) Fraction of mice surviving following treatment with PBS or virus. Symbols are as in *A*. One animal injected intratumorally with AdCAIL-2 died of unknown causes 6 days postinjection and is not included in the data set.

PBS controls, possibly a result of anti-adenoviral immune responses generated against virus-infected cells (46). However, all Addl70-3-injected tumors continued to enlarge until the mice became moribund (Fig. 4*B*). In contrast, eight of nine tumors injected with AdCAIL-2 underwent complete regression, and by 3–4 weeks postinjection neither visible nor palpable tumors could be detected. Mice in which complete tumor regression occurred remained tumor free for 12 weeks postvirus injection, at which time they were used in a challenge experiment (see below). In the one mouse in which tumor regression failed to occur (dashed line in Fig. 4*A*), the kinetics of tumor growth was not significantly different from that of the PBS control. This could reflect a failure to deliver virus efficiently into the tumor of this mouse or a subsequent leak of virus out of the site of injection.

Tumor-bearing animals that were injected with the same dose of virus s.c. in the flank opposite to that of the tumor failed to show any tumor regression or growth delay (data not shown), suggesting that IL-2 must be delivered intratumorally to mediate the effective immune responses. These results support the hypothesis that TILs within the tumor mass become activated and subsequently kill the tumor cells mediating regression. Histological sections of tumors 7 days postinjection with AdCAIL-2 showed a marked increase in lymphocytes present within the tumor compared to sections from control tumors, suggesting that IL-2 induces proliferation and accumulation of lymphocytes in the tumor (data not shown).

Results from the experiment of Fig. 4 and two additional experiments are presented in Table 1. Although complete tumor regression was observed only in a minority of the animals treated in experiments 2 and 3, a pronounced delay in tumor growth was observed in most animals (87% for pooled data from all three experiments), and 54% of AdCAIL-2-treated tumors completely regressed. In animals showing a partial response, tumor development was significantly delayed and survival was increased for up to 6 weeks longer than for untreated animals or control virus-injected animals. Factors that might contribute to the variability in the response to AdCAIL-2 injection are the efficiency of delivery of the transducing virus to the tumor and its retention and dissemination within the tumor mass. In addition, the tumor cells are derived from different individual transgenic mice for each experiment and the variation noted between experiments may be due to differences in immune susceptibility of the tumor cells obtained from different animals.

AdCAIL-2-Treated Mice Are Protected from Tumor Challenge. It was important to determine whether mice in which tumors regressed were resistant to a second tumor cell challenge. Mice that underwent complete tumor regression were challenged with freshly isolated tumor cells 16 weeks postvirus injection (12 weeks after complete regression of primary tumors) by injection of 10^6 cells s.c. into the opposite flank. Untreated age-matched control animals were also injected with the tumor cell suspension to ensure the cell preparation possessed the potential to form solid tumors. All control mice (five in each experiment) developed tumors between days 15 and 21, whereas the experimental mice showed no sign of tumor development and are currently tumor free at 32 (eight mice) and 42 weeks (three mice) postchallenge in two independent experiments. Thus, mice in which tumors had regressed following AdCAIL-2 injection developed long-term immunity against a subsequent tumor challenge. This immunity is likely to be tumor specific, as it has been previously shown that animals that have been injected with IL-2-transduced tumor cells are protected from challenge with parental tumor cells but not from unrelated tumors (13, 23, 25).

Table 1. Tumor response following intratumoral injection of AdCAIL-2

	Response			
	Exp. 1	Exp. 2	Exp. 3	Total*
None†	1	2	0	3/24 (13)
Partial‡	0	2	6	8/24 (33)
Complete§	8	1	4	13/24 (54)

*Percentage in parentheses.
†Tumor growth not significantly different from that of animals treated with control Addl70-3.
‡Tumor growth significantly delayed and animals survived 2–6 wk longer than controls.
§Complete regression of tumors and no reoccurrence.

In view of the fact that AdCAIL-2 transduction of tumor cells prior to injection resulted only in a delay in tumor development, the potency of AdCAIL-2 when directly injected into existing tumors is surprising. It would appear that the time and mode of delivery of AdCAIL-2 may be critically important in mediating tumor regression. This may reflect a requirement for immune effector cells to be recruited to the tumor site, primed, and activated, by which time the immune response may be insufficient or too late to completely abolish tumor growth. In contrast, when animals are tumor bearing, sufficient time has passed for recruitment and priming of immune effector cells within the tumor prior to cytokine treatment, and thus intratumoral expression of IL-2 at this point may be much more effective, resulting in activation of effector cells and potent antitumor activity. Our results suggest that in the intratumoral injection experiments, a resident effector cell population is present but does not respond to the tumor load and mediate regression. The delivery of IL-2 into the tumor results in the activation of this cell population and induces the potent antitumor responses seen. That not all tumors regressed when treated in this way may be a reflection of the use of primary tumor cells, which, like tumors in a clinical setting, may vary in their susceptibility to therapy. It is important to note that mice injected s.c. with PyMidT tumor cells always develop tumors at the site of injection and that the tumors have the potential to metastasize and never regress spontaneously. Thus ability to obtain regression of a majority of tumors by AdCAIL-2 administration is highly significant.

Fearon *et al.* (13) demonstrated that tumor cells secreting IL-2 could induce an antitumor activity that was dependent on $CD8^+$ T cells and that this treatment could induce protective immunity. However, they found that this protection was short lived and that 50% of animals challenged 4 weeks after immunization developed tumors. In contrast, our findings that "cured" mice were protected against a challenge 3 months after primary tumor regression strongly suggest that these mice had developed a long-lasting immunity to the PyMidT tumor cells. Unlike cancer therapies that use cytotoxic genes or drugs to mediate tumor cell killing (47–50), this form of immunotherapy may generate immune responses that would lead to protection from distal secondary tumor development or metastases. Development of this form of immunity is extremely important for the treatment of cancers such as breast cancer and melanoma, where the patient usually succumbs to the metastatic spread of the disease. Recently, Haddada *et al.* (28) and Cordier *et al.* (50) have also reported a therapeutic effect of an Ad vector carrying the murine IL-2 gene in the P815 mastocytoma model. Our studies provide further evidence that the delivery of Ad vectors carrying cytokine genes can induce highly effective antitumor responses and long-lasting immunity in a primary tumor model and may therefore be useful in establishing protective immunity in patients, thus preventing the metastatic spread of the cancer.

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